



FINAL REPORT

Test Facility Study No. 511875

Assessment of Skin Sensitization to MLA-3202 in the Mouse (Local Lymph Node Assay)

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1. STATEMENT OF GLP COMPLIANCE

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands

All phases of this study performed by the test facility were conducted in compliance with the following GLP regulations:

- OECD Principles of Good Laboratory Practice concerning Mutual Acceptance of Data in the Assessment of Chemicals, 26 November 1997 (C(97) 186 Final);
- EC Council Directive 2004 (2004/10/EC, February 11, 2004, Official Journal of February 20, 2004).

Except for the following:

Trial formulation preparation (for optimal vehicle selection) had a non-GLP status but was carried out in the quality assured environment of Charles River Den Bosch GLP test facility.

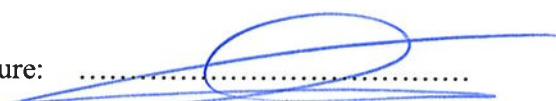
The test item characterization information supplied by the sponsor was produced under the sponsor's quality system.

Analysis of test item in vehicle for concentration, stability, homogeneity was not performed, however, to limit the impact, the test item preparation was performed with approved procedures and documented in detail. Preparations were visually inspected for homogeneity prior to use and all preparations were used within 4 hours after adding the vehicle to the test item of the formulation.

The data generated and reported are considered to be valid.

Charles River Den Bosch

Signature:



Name: A.H.B.M. van Huygevoort, MSc.

Title: Study Director

Date: 03 October 2016

2. TEST FACILITY QUALITY ASSURANCE STATEMENT

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands.

Study title: Assessment of skin sensitization to MLA-3202 in the mouse (local lymph node assay)

This report was inspected by the Charles River Den Bosch Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s). The reported method and procedures were found to describe those used and the report reflects the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected. The dates of Quality Assurance inspections are given below.

Project	511875	Start Inspection date	End Inspection date	Reporting date
Type of Inspections	Phase/Process			
Study	Study Plan Report	22-Apr-2016 04-Jul-2016	22-Apr-2016 04-Jul-2016	22-Apr-2016 04-Jul-2016
Process	Analytical and physical chemistry Test Substance Handling Exposure Observations/Measurements Specimen Handling	01-Mar-2016	11-Mar-2016	14-Mar-2016
	Animal Facilities Test Substance Handling Exposure Observations/Measurements Specimen Handling	04-Apr-2016	15-Apr-2016	15-Apr-2016
	Test Substance Receipt Test Substance Handling	09-May-2016	20-May-2016	24-May-2016
	Test Substance Formulation Test Substance Handling	30-May-2016	13-Jun-2016	14-Jun-2016

The review of the final report was completed on the date of signing this QA statement.

Charles River Den Bosch

Signature: Ali Bouhuijzen

Name: Ali Bouhuijzen M. Sc.
Compliance Specialist III

Date: 27-Sep-2016

3. SUMMARY

Assessment of skin sensitization to MLA-3202 in the Mouse (Local Lymph Node Assay).

The study was carried out based on the guidelines described in:
OECD, Section 4, Health Effects, No.429 (2010),
EC, No 440/2008; B42: "Skin Sensitization: Local Lymph Node Assay"
EPA, OPPTS 870.2600 (2003) "Skin Sensitization".

Test item concentrations selected for the main study were based on the results of a pre-screen test.

In the main study, three experimental groups of five female CBA/J mice were treated with test item concentrations of 10, 25 or 50% w/w on three consecutive days, by open application on the ears. Five vehicle control animals were similarly treated, but with the vehicle alone (Acetone/Olive oil (4:1 v/v)). Three days after the last exposure, all animals were injected with ³H-methyl thymidine and after five hours the draining (auricular) lymph nodes were excised and pooled for each animal. After precipitating the DNA of the lymph node cells, radioactivity measurements were performed. The activity was expressed as the number of disintegrations per minute (DPM) and a stimulation index (SI) was subsequently calculated for each group.

No erythema of the ears was noted for any of the animals. Scaliness was noted for the ears of the animals dosed at 25 and 50% on Day 6, which was considered not to have a toxicologically significant effect on the activity of the nodes.

No mortality occurred and no clinical signs of systemic toxicity were observed in the animals of the main study. Body weights and body weight gain of experimental animals remained in the same range as controls over the study period.

Enlarged auricular lymph nodes were found for the animals dosed at 50%. The nodes of the other dose groups and the control group were considered normal in size. No macroscopic abnormalities of the surrounding area were noted for any of the animals.

Mean DPM/animal values for the experimental groups treated with test item concentrations 10, 25 and 50% were 822, 1219 and 3222 DPM, respectively. The mean DPM/animal value for the vehicle control group was 531 DPM. The SI values calculated for the test item concentrations 10, 25 and 50% were 1.5, 2.3 and 6.1, respectively

These results indicate that the test item could elicit a $SI \geq 3$. The data showed a dose-response and an EC₃ value (the estimated test item concentration that will give a $SI = 3$) of 30% was calculated.

The six-month reliability check with Alpha-hexylcinnamaldehyde indicates that the Local Lymph Node Assay as performed at Charles River Den Bosch is an appropriate model for testing for contact hypersensitivity.

However, additional information was provided by the Sponsor stating that “following the LLNA study, the surface tension study for MLA-3202 showed it to be a surface active substance”. The OECD guideline state that surface active substances have been found to cause false positives in the LLNA study. Therefore, it cannot be excluded that a non-specific stimulation of the lymph nodes occurred, not related to skin sensitization.

Based on these results and evaluation and according to the recommendations made in the test guidelines (including all amendments), MLA-3202 might be regarded as skin sensitizer. However, a false positive outcome cannot be excluded.

4. INTRODUCTION

Due to the acquisition of WIL Research by Charles River, the name of the WIL Research facility in Den Bosch, has been changed to Charles River Laboratories Den Bosch BV, Hambakenwetering 7, 5231 DD Den Bosch, The Netherlands. Study documents may contain both names and both names are considered equivalent and may be used as the name of WIL Research transitions to Charles River.

4.1. Study Schedule

Experimental starting date : 27 April 2016
Experimental completion date : 30 May 2016

4.2. Purpose

The purpose of this study was to evaluate whether the test item induces skin sensitization in mice after three epidermal induction exposures of the animals under the conditions described in this report. This study should provide a rational basis for risk assessment in man. Compared to sensitization tests using guinea pigs, the local lymph node assay (LLNA) provides advantages with regard to animal welfare and scientific aspects.

4.3. Guidelines

This type of study plan was reviewed and agreed by the Laboratory Animal Welfare Officer and the Ethical Committee (DEC 14-23) as required by the Dutch Act on Animal Experimentation (February 1997).

The study procedures described in this report were in compliance with the following guidelines:

- Organization for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.429, "Skin Sensitization: Local Lymph Node Assay", Paris Cedex, July 2010.
- Commission Regulation (EC) No 440/2008 Part B: Methods for the Determination of Toxicity and other Health Effects; B42: "Skin Sensitization: Local Lymph Node Assay". Official Journal of the European Union No. L142, May 2008, including most recent amendments.
- Environmental Protection Agency (EPA): Health Effects Test Guidelines OPPTS 870.2600. "Skin Sensitization", March 2003.

4.4. Retention of Records and Materials

Records and material pertaining to the study, which include study plan and amendments, raw data, specimens, except perishable specimens, and the final report will be retained in the archives of the test facility for a minimum of 5 years after the finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. The test facility will retain information concerning decisions made.

A sample of the test item will be retained until expiry date or applicable retest date. After this period the sample(s) will be destroyed.

4.5. Responsible Personnel**4.5.1. Test Facility**

Study Director A.H.B.M. van Huygevoort, MSc.

Coordinating Biotechnician M.R.M. Blonk (Charles River Den Bosch)

QA C.J. Mitchell, BSc. (Charles River Den Bosch)

Test Facility Management H.H. Emmen, MSc. (Charles River Den Bosch)
Representative

4.5.2. Sponsor Representative

Study Monitor Audrey Batoon, Ph.D.

5. MATERIALS AND METHODS

5.1. Test Item

5.1.1. Test Item Information

Test item	207258/A
Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

5.1.2. Study Specific Test Item Information

Purity/composition correction factor	No correction factor required
Test item handling	No specific handling conditions required
Stability at higher temperatures	Stable
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3
pH	6-7
Specific gravity/density	0.9394

5.1.3. Test Item Preparation

Vehicle	Acetone/Olive oil (4:1 v/v) (Acetone p.a.: Merck, Darmstadt, Germany; Olive oil: Fagron, Nieuwerkerk a/d IJssel, The Netherlands).
Rationale	The vehicle was selected on the basis of maximizing the solubility using the test item data provided by the Sponsor and trial preparation results performed at Charles River Den Bosch. The vehicle was chosen from the vehicles specified in the test guideline: Acetone/Olive oil (4:1 v/v), N,N-dimethylformamide, methylethylketone, propylene glycol, dimethylsulfoxide . There was no information available regarding the solubility or stability in vehicle.
Preparation	The test item preparations (w/w) were prepared within 4 hours prior to each dosing. No adjustment was made for specific gravity of the vehicle. Homogeneity was assessed by visual inspection of the solutions.

Correction of the purity/composition of the test item is not applicable, since the test method requires a logical concentration range rather than specific dose levels to be dosed.

5.2. Test System

Species	Mouse, CBA/J strain, inbred, SPF-Quality. Recognized by the international guidelines as the recommended test system (e.g. OECD, EC, EPA). Source: Janvier, Le Genest-Saint-Isle, France
Number of animals	20 females (nulliparous and non-pregnant), five females per group (main study only).
Age and body weight	Young adult animals (approx. 10 weeks old) were selected. Body weight variation was within +/- 20% of the sex mean.
Identification	Tail mark with a marker pen.
Health inspection	At least prior to dosing. It was ensured that the animals were healthy and that the ears were intact and free from any abnormality.
Reliability check	The results of a reliability test with three concentrations of Hexylcinnamaldehyde (CAS No. 101-86-0) in Acetone/Olive oil (4:1 v/v), performed not more than 6 months previously and using the same materials, animal supplier, animal strain and essential procedures are summarized in APPENDIX 2 of this report. For both scientific and animal welfare reasons, no concurrent positive control group was included in the study. An extensive data base is available with reliability checks performed at half year intervals during at least the past 9 years showing reproducible and consistent positive results.

5.3. Animal Husbandry

Conditions

Environmental controls for the animal room were set to maintain 18 to 24°C, a relative humidity of 40 to 70%, at least 10 air changes/hour, and a 12-hour light/12-hour dark cycle. Any variations to these conditions were maintained in the raw data and had no effect on the outcome of the study.

Accommodation

Animals were group housed in labeled Makrolon cages (MIII type; height 18 cm) containing sterilized sawdust as bedding material (Lignocel S 8-15, JRS - J.Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany). Paper (Enviro-dri, Wm. Lillico & Son (Wonham Mill Ltd), Surrey, United Kingdom) and shelters (disposable paper corner home, MCORN 404, Datesand Ltd, USA) were supplied as cage-enrichment. The acclimatization period was at least 5 days before the start of treatment under laboratory conditions. On Day 6, the animals were group housed in Makrolon MII type cages with a sheet of paper instead of sawdust and cage enrichment.

Diet

Free access to pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany).

Water

Free access to tap water.

Diet, water, bedding and cage enrichment evaluations for contaminants and/or nutrients were performed according to facility standard procedures. There were no findings that could interfere with the study.

5.4. Weight of Evidence Analysis

In the interest of animal welfare and to minimize any testing likely to produce severe responses in animals, a weight of evidence analysis was performed prior to the start of this study. All available information was evaluated (e.g. existing human and animal data, literature, item data supplied by the Sponsor, analysis of structure activity relationships (SAR), physicochemical properties and reactivity (pH, buffering capacity)). It was concluded by the Study Director that no severe effects were to be expected.

5.5. Pre-screen Test

A pre-screen test was conducted in order to select the highest test item concentration to be used in the main study. In principle, this highest concentration should cause no systemic toxicity, may give well-defined irritation as the most pronounced response (maximum grade 2 and/or an increase in ear thickness < 25%) and/or is the highest possible concentration that can technically be applied.

Two test item concentrations were tested; a 100% and 50% concentration. The highest concentration was the maximum concentration as required in the test guidelines.

The test system, procedures and techniques were identical to those used in the main study except that the application method may have been different (see tables) and that the assessment of lymph node proliferation and necropsy were not performed. Two young adult animals per concentration were selected. Each animal was treated with one concentration on three consecutive days. Animals were group housed in labeled Makrolon cages (MII type, height 14 cm). Ear thickness measurements were conducted using a digital thickness gauge (Kroeplin C110T-K) prior to dosing on Days 1 and 3, and on Day 6. Animals were sacrificed after the final observation.

5.6. Main Study

Three groups of five animals were treated with one test item concentration per group. The highest test item concentration was selected from the pre-screen test.
One group of five animals was treated with the vehicle.

5.6.1. Allocation

Group ¹	animal numbers	induction (test item; % w/w)
1	01 - 05	0 (Acetone/Olive oil (4:1 v/v))
2	06 - 10	10
3	11 - 15	25
4	16 - 20	50

¹. five females per group

5.6.2. Induction - Days 1, 2 and 3

The dorsal surface of both ears was topically treated (25 µL/ear) with the test item, at approximately the same time on each day. The concentrations were stirred with a magnetic stirrer immediately prior to dosing.

The control animals were treated in the same way as the experimental animals, except that the vehicle was administered instead of the test item.

5.6.3. Excision of the Nodes - Day 6

Each animal was injected via the tail vein with 0.25 mL of sterile phosphate buffered saline (PBS) (Merck, Darmstadt, Germany) containing 20 µCi of ³H-methyl thymidine (PerkinElmer Life and Analytical Sciences, Boston, MA, US).

After five hours, all animals were killed by intraperitoneal injection (0.2 mL/animal) of Euthasol® 20% (AST Farma BV, Oudewater, The Netherlands). The draining (auricular) lymph node of each ear was excised. The relative size of the nodes (as compared to normal) was estimated by visual examination and abnormalities of the nodes and surrounding area were recorded. The nodes were pooled for each animal in approximately 3 mL PBS.

5.6.4. Tissue Processing for Radioactivity - Day 6

Following excision of the nodes, a single cell suspension of lymph node cells (LNC) was prepared in PBS by gentle separation through stainless steel gauze (diameter: 125 µm). LNC were washed twice with an excess of PBS by centrifugation at 200g for 10 minutes at 4°C. To precipitate the DNA, the LNC were exposed to 5% trichloroacetic acid (TCA) (Merck, Darmstadt, Germany) and then stored in the refrigerator until the next day.

5.6.5. Radioactivity Measurements - Day 7

Precipitates were recovered by centrifugation, resuspended in 1 mL TCA and transferred to 10 mL of Ultima Gold cocktail (PerkinElmer Life and Analytical Sciences, Boston, MA, US) as the scintillation fluid. Radioactivity measurements were performed using a Packard scintillation counter (2800TR). Counting time was to a statistical precision of ± 0.2% or a maximum of 5 minutes whichever came first. The scintillation counter was programmed to automatically subtract background and convert Counts Per Minute (CPM) to Disintegrations Per Minute (DPM).

5.7. Observations

Mortality/Viability	Twice daily.
Body weights	On Day 1 (pre-dose) and Day 6 (prior to necropsy).
Clinical signs	Once daily on Days 1-6 (on Days 1-3 between 3 and 4 hours after dosing).
Irritation	Once daily on Days 1-6 (on Days 1-3 within 1 hour after dosing) according to the following numerical scoring system. In addition, a description of all other (local) effects was recorded.

Grading Irritation Reactions:

Erythema and eschar formation:

No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema (beet redness) to slight eschar formation (injuries in depth) ...	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

Necropsy No necropsy for gross macroscopic examination was performed according to study plan.

5.8. Interpretation

DPM values are presented for each animal and for each dose group. A Stimulation Index (SI) is calculated for each group using the individual SI values. The individual SI is the ratio of the DPM/animal compared to the DPM/vehicle control group mean.

If the results indicate a $SI \geq 3$, the test item may be regarded as a skin sensitizer.

The EC3 value (the estimated test item concentration that will give a $SI = 3$) was determined, using linear interpolation ([Ref. 1](#)).

5.9. List of Deviations**5.9.1. List of Study Plan Deviations**

There were no deviations from the study plan.

5.9.2. List of Standard Operating Procedures Deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

6. ELECTRONIC SYSTEMS FOR DATA ACQUISITION

The following electronic systems were used for data acquisition:

REES Centron Environmental Monitoring system version SQL 2.0 (REES scientific, Trenton, NJ, USA); Quantasmart 2.03 (PerkinElmer Life Sciences, Boston, MA, USA): LSC software.

7. RESULTS

For detailed results see [APPENDIX 1: TABLES AND FIGURES](#)

7.1. Pre-screen Test

At a 100% test item concentration, signs of systemic toxicity (piloerection and hunched posture) were noted indicating that this concentration did not comply with the selection criteria. At 50%, no systemic toxicity and very slight erythema and scaliness for the ears were observed. Variations in ear thickness during the observation period were less than 25% from Day 1 pre-dose values. Transparent test item remnants were present on the dorsal surface of the ears.

Based on these results, the highest test item concentration selected for the main study was a 50% concentration.

7.2. Main Study

7.2.1. Skin Reactions / Irritation

No erythema of the ears was noted for any of the animals. Scaliness was noted for the ears of the animals dosed at 25 and 50% on Day 6, which was considered not to have a toxicologically significant effect on the activity of the nodes.

7.2.2. Systemic Toxicity

No mortality occurred and no clinical signs of systemic toxicity were observed in the animals of the main study. Body weights and body weight gain of experimental animals remained in the same range as controls over the study period.

7.2.3. Macroscopic Examination of the Auricular Lymph Nodes and Surrounding Area

Enlarged auricular lymph nodes were found for the animals dosed at 50%. The nodes of the other dose groups and the control group were considered normal in size. No macroscopic abnormalities of the surrounding area were noted for any of the animals.

7.2.4. Radioactivity Measurements and SI Values

Mean DPM/animal values for the experimental groups treated with test item concentrations 10, 25 and 50% were 822, 1219 and 3222 DPM, respectively. The mean DPM/animal value for the vehicle control group was 531 DPM. The SI values calculated for the test item concentrations 10, 25 and 50% were 1.5, 2.3 and 6.1, respectively.

8. CONCLUSION

These results indicate that the test item could elicit a $SI \geq 3$. The data showed a dose-response and an EC₃ value (the estimated test item concentration that will give a $SI = 3$) of 30% was calculated.

The six-month reliability check with Alpha-hexylcinnamaldehyde indicates that the Local Lymph Node Assay as performed at Charles River Den Bosch is an appropriate model for testing for contact hypersensitivity (see [APPENDIX 2](#)).

However, additional information was provided by the Sponsor stating that “following the LLNA study, the surface tension study for MLA-3202 showed it to be a surface active substance”. The OECD guideline state that surface active substances have been found to cause false positives in the LLNA study. Therefore, it cannot be excluded that a non-specific stimulation of the lymph nodes occurred, not related to skin sensitization.

Based on these results and evaluation and according to the recommendations made in the test guidelines (including all amendments), MLA-3202 might be regarded as skin sensitizer. However, a false positive outcome cannot be excluded.

9. REFERENCES

- Ref. 1 Baskett DA, Lea LJ, Dickens A, Briggs, D, Pate I, Dearman RJ and Kimber I. A comparison of statistical approaches to the derivation of EC₃ values from local lymph node assay dose responses. *J Appl Toxicol* 1999;19:261-266.

APPENDIX 1: TABLES AND FIGURES

PRE-SCREEN TEST**Table 1: Body Weights and Skin Reactions**

TS ¹ (%)	animal	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		
		bw (g) ²	erythema ³	left	right	erythema	left	right	erythema	left	right	erythema	left	bw (g)
50	1	21.3	0	0	0	0	1f	1	1	1	0	0	0	21.4
	2	22.0	0	0	0	0	1f	1f	1f	1fs	0	1s	0s	22.4
100 ⁴	3	22.3	0f	0f	1f	0f	2f	1f	1fs	1f	1s	1s	1s	22.7
	4	23.1	0f	0f	0f	1f	1f	1f	1fs	1f	1s	1s	1s	23.4

s. Scaliness, f. transparent test item remnants on the dorsal surface of the ears

¹. TS = test item (% w/w).². Body weight (grams).³. Grading erythema and eschar formation (Left = dorsal surface of left ear; right = dorsal surface of right ear):

0 = No erythema

1 = Very slight erythema (barely perceptible)

2 = Well-defined erythema

⁴. Applied using a pipette with the tip cut off.

Note 1: Red skin was noted between the ears of all animals on day 3 and for the animals dosed at 100% on Days 4, 5 and 6.

Note 2: At 100%, piloerection and hunched posture were seen on Day 3 and piloerection for animal 4 on Day 4.

Table 2: Ear Thickness Measurements

TS ¹ (%)	Animal	Day 1		Day 3				Day 6			
		left (mm)	right (mm)	left (mm)	% ²	right (mm)	% ²	left (mm)	% ²	right (mm)	% ²
50	1	0.230	0.225	0.235	2	0.235	4	0.240	4	0.235	4
	2	0.220	0.220	0.230	5	0.235	7	0.245	11	0.235	7
100	3	0.220	0.220	0.250	14	0.245	11	0.260	18	0.255	16
	4	0.225	0.225	0.240	7	0.245	9	0.250	11	0.255	13

Left (mm) = thickness of left ear in millimetres; right (mm) = thickness of right ear in millimetres.

¹. TS = test item (% w/w).². Percent increase compared to Day 1 pre-dose value.

MAIN STUDY**Table 3: Body Weights and Skin Reactions**

group	TS ¹ (%)	animal	Day 1			Day 2		Day 3		Day 4		Day 5		Day 6	
			bw (g) ²	erythema ³ left	erythema ³ right	erythema Left	erythema right	erythema left	erythema right	erythema left	erythema right	erythema left	erythema right	bw (g)	
1	0	1	21.8	0	0	0	0	0	0	0	0	0	0	0	21.3
		2	22.1	0	0	0	0	0	0	0	0	0	0	0	22.4
		3	22.0	0	0	0	0	0	0	0	0	0	0	0	22.2
		4	20.2	0	0	0	0	0	0	0	0	0	0	0	21.7
		5	22.1	0	0	0	0	0	0	0	0	0	0	0	21.5
2	10	6	22.4	0	0	0	0	0	0	0	0	0	0	0	23.4
		7	21.1	0	0	0	0	0	0	0	0	0	0	0	21.4
		8	22.9	0	0	0	0	0	0	0	0	0	0	0	21.8
		9	22.2	0	0	0	0	0	0	0	0	0	0	0	21.9
		10	21.0	0	0	0	0	0	0	0	0	0	0	0	20.8
3	25	11	21.6	0	0	0	0	0	0	0	0	0	0	0	21.3
		12	22.3	0	0	0	0	0	0	0	0	0	0	0s	23.0
		13	18.5	0	0	0	0	0	0	0	0	0	0s	0	18.3
		14	22.1	0	0	0	0	0	0	0	0	0	0	0	22.6
		15	22.2	0	0	0	0	0	0	0	0	0	0s	0s	21.4
4	50	16	22.3	0	0	0	0	0	0	0	0	0	0s	0s	24.3
		17	21.0	0	0	0	0	0	0	0	0	0	0s	0s	20.4
		18	22.5	0	0	0	0	0	0	0	0	0	0s	0s	23.3
		19	21.2	0	0	0	0	0	0	0	0	0	0s	0	21.2
		20	21.5	0	0	0	0	0	0	0	0	0	0s	0	21.5

s. Scaliness

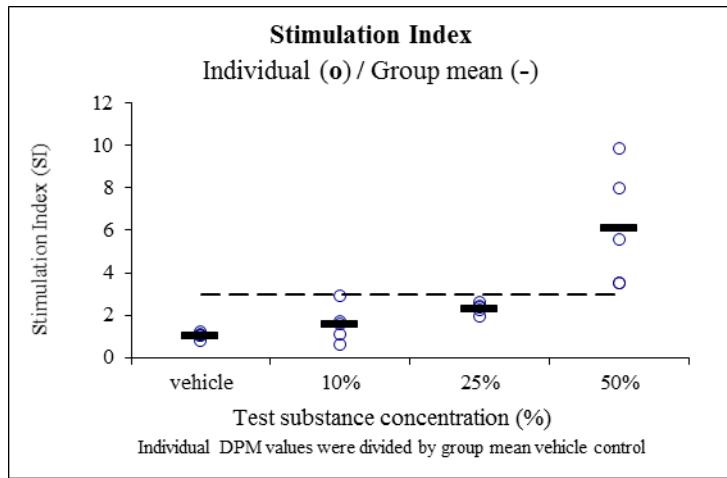
¹. TS = test item (% w/w).². Body weight (grams).³. Grading erythema and eschar formation (Left = dorsal surface of left ear; right = dorsal surface of right ear):

0 = No erythema

MAIN STUDY**Table 4: Relative Size Lymph Nodes, Radioactivity Counts (DPM) and Stimulation Index (SI)**

group	TS ¹ (%)	animal	Size nodes ²		DPM ³ / animal	mean DPM ± SEM ⁴	mean SI ± SEM
			left	right			
1	0	1	n	n	622		
		2	n	n	527		
		3	n	n	536	531 ± 37	1.0 ± 0.1
		4	n	n	400		
		5	n	n	572		
2	10	6	n	n	873		
		7	n	n	1542		
		8	n	n	833	822 ± 207	1.5 ± 0.4
		9	n	n	551		
		10	n	n	311		
3	25	11	n	n	1024		
		12	n	n	1376		
		13	n	n	1271	1219 ± 58	2.3 ± 0.2
		14	n	n	1243		
		15	n	n	1181		
4	50	16	n	n	5226		
		17	n	+	4232		
		18	n	+	1846	3222 ± 665	6.1 ± 1.3
		19	n	n	1862		
		20	n	n	2946		

¹. TS = test item (% w/w).². Relative size auricular lymph nodes (-, -- or ---: degree of reduction, +,++ or +++: degree of enlargement, n: considered to be normal).³. DPM = Disintegrations per minute⁴. SEM = Standard Error of the Mean

Figure 1: Dose-response Curve

APPENDIX 2: RELIABILITY CHECK

ASSESSMENT OF CONTACT HYPERSENSITIVITY

TO ALPHA- HEXYL CINNAMALDEHYDE, TECHNICAL GRADE

IN THE MOUSE (LOCAL LYMPH NODE ASSAY)

A RELIABILITY CHECK.

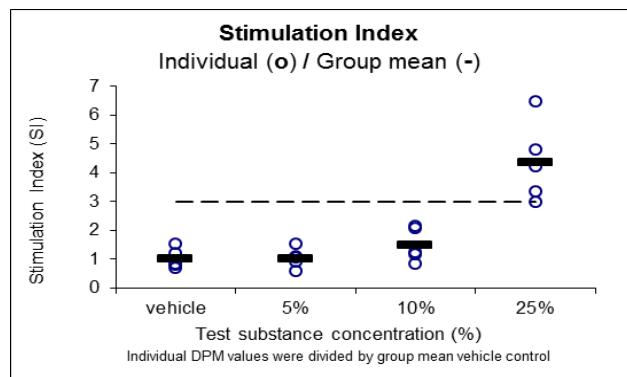
Test Facility Study No. 511360

SUMMARY RELIABILITY CHECK

A reliability check is carried out at regular intervals to check the sensitivity of the test system and the reliability of the experimental techniques as used by Charles River Den Bosch. In this study, performed in November 2015, females of the CBA/J mouse strain (Janvier, Le Genest-Saint-Isle, France) were checked for sensitivity to Hexylcinnamaldehyde. The females were approx. 10 weeks old at commencement of the study. The study was based on the OECD Guideline No. 429, EC No 440/2008, Part B.42 and EPA, OPPTS 870.2600 "Skin Sensitization". Alpha- Hexylcinnamaldehyde, technical grade (CAS no. 101-86-0) was fabricated under lot no. MKBJ8846V (Sigma- Aldrich, Steinheim, Germany). Concentrations used for this study were 5, 10 and 25% in Acetone/Olive oil (4:1 v/v).

Group ¹	% Alpha- Hexylcinnamaldehyde, technical grade	mean		
		DPM ± SEM	SI ± SEM	
1	0% (Acetone:Olive oil (4:1 v/v))	1044 ± 160	1.0 ± 0.2	
2	5%	1062 ± 154	1.0 ± 0.2	
3	10%	1536 ± 275	1.5 ± 0.3	
4	25%	4551 ± 643	4.4 ± 0.9	

¹ Five females per group.

**CONCLUSION**

The SI values calculated for the item concentrations 5, 10 and 25% were 1.0, 1.5 and 4.4 respectively. An EC3 value of 17.8% was calculated using linear interpolation.

The calculated EC3 value was found to be in the acceptable range of 4.8 and 19.5%. The results of the 6 monthly HCA reliability checks of the recent years were 14.5, 13.4, 14.1, 17.3, 9.8% and 10%.

Based on the results, it was concluded that the Local Lymph Node Assay as performed at Charles River Den Bosch is an appropriate model for testing for contact hypersensitivity. The raw data, study plan and report from this study are kept in the Charles River Den Bosch archives. The test described above was performed in accordance with Charles River Den Bosch Standard Operating Procedures and the report was audited by the QA-unit.

APPENDIX 3: TEST ITEM CERTIFICATE OF ANALYSIS



Chemtura Corporation
12 Spencer St
Naugatuck, CT 06770

Analytical Services
www.chemtura.com

Certificate of Purity

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	C ₂₄ H ₄₇ NO ₃	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	C ₂₂ H ₄₅ NO ₃	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	C ₂₄ H ₄₅ NO ₃	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	C ₂₄ H ₄₉ NO ₃	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	C ₂₂ H ₄₃ NO ₃	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	C ₂₆ H ₄₅ NO ₃	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	C ₂₄ H ₄₃ NO ₃	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	C ₁₈ H ₃₄ O ₂	C18:1 (oleic) acid
1.1	421.4	C ₂₆ H ₄₇ NO ₃	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

Blake Lewis

3/7/16

Blake Lewis
Analytical REACH Scientist, Analytical Services

Date

Colin Moore

Son *AT&T*
Albert J. Nitowski
Sr. Technology Manager
Analytical and Lab Support Services

**APPENDIX 4: ENDORSEMENT OF COMPLIANCE WITH THE OECD
PRINCIPLES OF GLP**



ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on 7 – 11, 14 and 16 September 2015 at

WIL Research Europe B.V.
Hambakenwetering 7
5231 DD 's Hertogenbosch

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following area of expertise: physical-chemical testing, toxicity studies, mutagenicity studies, environmental toxicity studies on aquatic and terrestrial organisms, studies on behaviour in water, soil, and air, bioaccumulation, residue studies, analytical and clinical chemistry testing, kinetic and metabolism studies and safety pharmacology.

Utrecht, 3 November 2015



Health Care Inspectorate of the Ministry of Health, Welfare and Sport
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P.O. Box 2680, 3500 GR Utrecht, The Netherlands